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Feeding common carp *Cyprinus carpio* with β-glucan supplemented diet stimulates C-reactive protein and complement immune acute phase responses following PAMPs injection

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ABSTRACT

The effect of β-glucan as a feed additive on the serum and gene profile of C-reactive protein (CRP) and complement acute phase responses was ascertained in common carp *Cyprinus carpio*. In addition effects of subsequent intraperitoneal injections of pathogen-associated molecular patterns (PAMPs), i.e. LPS or poly(I:C), to mimic bacterial or viral infection respectively, were studied. Carp were first orally fed with β-glucan (MacroGard®) with a daily β-glucan intake of 6 mg per kg body weight or with control food for 25 days and then injected with PBS containing either LPS (4 mg/kg) or poly(I:C) (5 mg/kg) or PBS alone. Fish were sampled during the 25 days of the feeding period and up to 7 days post-PAMPs injections for serum and liver, head kidney and mid-gut tissues. Oral administration of β-glucan for 25 days significantly increased serum CRP levels and alternative complement activity (ACP). In addition, the subsequent LPS and poly(I:C) challenges significantly affected CRP and complement related gene expression profiles (*crp1*, *crp2*, *c1r/s*, *bf/c2*, *c3* and *masp2*), with the greatest effects observed in the β-glucan fed fish. However, in fish fed β-glucan the PAMPs injections had less effects on CRP levels and complement activity in the serum than in control fed fish, suggesting that the 25 days of β-glucan immunostimulation was sufficient enough to reduce the effects of LPS and poly(I:C) injections. Results suggest that MacroGard® stimulated CRP and complement responses to PAMPs immunological challenges in common carp thus highlighting the beneficial β-glucan immunostimulant properties.

1. Introduction

Fish mortalities resulting from transportation, high stocking densities, pathogen outbreaks or spawning are important in the economic viability of aquaculture. Although prophylactic measures such as treatment with antibiotics and vaccine administration have been adopted widely to reduce fish losses these control strategies have either possible environmental impacts or are limited by their specificity [1]. An alternative and promising disease prevention method used in fish farming is the enhancement of the fish innate immune defenses through the administration of natural immunostimulants such as β-glucans [2] or other pathogen-associated molecular patterns (PAMPs) including lipopolysaccharide (LPS) [3] and polyinosinic:polycytidylic acid (poly(I:C)) [4] which mimic bacterial and viral infections respectively.

Although β-glucan, LPS and poly(I:C) have very different molecular structures, they share similar biological activities and mechanisms of action, for example the stimulation of the fish immune acute phase response (APR). β-Glucans, either administered through intraperitoneal injection or orally, can affect several APR parameters in fish such as increased lysozyme activity [5–8], respiratory burst activity [5,7,9–12], phagocytic activity [5,12–14], granulocyte number [6,9,10,14,15] and complement activity [6,16,17]. However, these beneficial effects are strongly dependent on the dose of β-glucan, the duration of the treatment and the route of administration [10]. The greatest effects are often observed when low doses of β-glucan are administered, e.g. 0.1% [5,18,19]. The endotoxin LPS is derived from prokaryotes and represents one of the major interaction sites between Gram negative bacteria and the antibacterial elements of the host's immune system [20]. LPS has an immunostimulating effect on several fish species [21–24], for

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example, an increase in production of cytokines such as IL-1β, IL-6 and TNF-α in the Atlantic salmon *Salmo salar* [25]. In contrast to the natural compounds β-glucans and LPS, poly(I:C) is a synthetic mismatched double stranded RNA comprising an inosinic acid polymer and a cytidylic acid polymer strands, which has been used in several studies to mimic RNA viral infection [4].

Previous studies [17,26] have revealed that bacterial and viral infections in common carp (*Cyprinus carpio*) invoke an APR. For example, serum levels of CRP increased up to 6 fold within 40 h of infection with *Aeromonas hydrophila* [25], and up to 10 fold within 72 h infection with CyHV-3 [personal observation]. In addition, complement activity is also significantly increased in common carp orally stimulated with β-glucan and challenged with *Aeromonas salmonicida* [17] or challenged with the CyHV3 virus without β-glucan stimulation [27]. This study aims to investigate how oral administration of the immunostimulant β-glucan affects the APR in carp induced by LPS and poly(I:C) intraperitoneal injections as mimics of bacterial and viral infections respectively.

2. Material and methods

2.1. Experimental animals

Common carp (*C. carpio*) with an average weight of 40 g were supplied by Fair Fisheries, UK and kept in individual tanks at Keele University, UK. 120 carp were divided in two tanks and maintained in 16 °C water under a 12/12 h light/dark cycle and acclimatised to the re-circulating system for 3 weeks, during which they were fed 1% body weight per day with a control diet lacking β-glucan (formulated and provided by Tetra GmbH, Germany).

2.2. Experimental diet formulation and feeding regimes

Experimental diet, also formulated and supplied by Tetra GmbH, comprised a standard pellet feed form fortified with 0.1% Macro-Gard®, a β-glucan source, supplied by Biorigin, Brazil (for details of food composition see [28]). Following the 3 week acclimatisation phase, one group of fish was kept on the control diet (lacking MacroGard®) while the other group was fed with the experimental diet (0.1% MacroGard®). Both feeding regimes were carried out at 1% fish body weight per day for 25 days and therefore fish fed with the experimental diet received a daily dose of 10 mg/kg body weight MacroGard® i.e recommended dose by the commercial supplier (Biorigin) corresponding to a daily intake of 6 mg β-glucan per kg of fish body weight as MacroGard® [28]. Fish which were fed with this MacroGard® supplemented diet are herein reported as "β-glucan fed fish".

2.3. Fish sampling during the 25 days of feeding

Five individuals from each group of fish were killed with a lethal dose of 2-Phenoxyethanol (Sigma Aldrich, UK) after 1, 3, 7 and 25 days of control or β-glucan feeding. Blood was taken from the caudal vein, allowed to clot overnight at 4 °C, centrifuged (2500 × *g* for 10 min at 4 °C) and the serum stored at −80 °C. Head kidney, mid-gut (in the gastric area) and liver tissues were taken after 7 and 25 days of feeding and stored in RNA later (Invitrogen) at −80 °C.

2.4. PAMPs (LPS and poly(I:C)) injections

After 25 days of feeding with a control or β-glucan diet, remaining fish were allocated to three different treatment groups within their respective feeding regime and received either an intraperitoneal injection of 100 μL PBS containing either LPS (4 mg/kg), poly(I:C) at 5 mg/kg or PBS alone. LPS from *Escherichia coli* 0111:B4 and poly(I:C) were purchased from Invivogen, UK. Six treatment groups were therefore established: control fed + PBS injected fish, control fed + LPS injected fish, control fed + poly(I:C) injected fish, β-glucan fed + PBS injected fish, β-glucan fed + LPS injected fish and β-glucan fed + poly(I:C) injected fish. Carp were fed their respective diets at 1% fish body weight per day. At 1, 3 and 7 days post-injection (p.i.) four fish per treatment were killed with 2-phenoxyethanol. Serum was collected at each time point and head kidney, mid-gut and liver tissues were removed and stored in RNAlater (Invitrogen) at −80 °C.

2.5. Quantification of free-phase carp CRP (cCRP) in serum

Serum free-phase carp CRP (cCRP) was quantified as previously described by Pionnier et al. [17] through a competitive enzyme-linked immunosorbent assay (ELISA) using CRP extracted and purified from serum of healthy common carp with a two-step affinity chromatography procedure developed by Cartwright et al. [29] and MacCarthy et al. [26]. Briefly the ELISA procedure comprised individual wells of a high-binding capacity 96-well plate (Corning) which had been coated with 0.2 μg of cCRP diluted in coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6) and incubated 20 h at 4 °C. Unbound cCRP was removed by a 3 times wash in a PBS-T buffer (PBS with 0.2% Tween-20) and the plate was blocked with PBS containing 5% of dried milk powder (Marvel) for 30 min at 37 °C. After a 3 times wash in a PBS-T buffer, carp serum (diluted 1:10 in PBS), which had been incubated with 160 μg/μL of rabbit anti-cCRP IgG for 30 min at 20 °C, was added. After incubation, 100 μL of the treated rabbit anti-cCRP IgG solution was applied to the cCRP-coated wells and the plate was incubated for 1 h at 37 °C. Excess antibody was removed by washing 3 times with PBS-T and then the secondary antibody (100 μL of goat anti-rabbit IgG peroxidase conjugate; Sigma, dilution 1:8000 in PBS), was applied to the wells for 1 h at 37 °C. After a final 5 times PBS-T wash, each well received 100 μL of ophenylenediamine dihydrochloride (Sigma). The plate was incubated for 1 h at 37 °C in the dark after which the enzymatic reaction was stopped with 25 μL of 2.5 M HCl, and the optical density was read at 492 nm (Labsystems Multiskan MS plate reader). The cCRP concentration of the individual serum samples was determined with the GraphPad Prism v5 software comparing the sample absorbance value to those from the standard curve for which the free phase cCRP concentration is known.

2.6. Alternative complement pathway activity assay in carp serum

Serum alternative complement pathway (ACP) activity was assayed according to previously published methods [9,30–32] with slight modifications as described by Pionnier et al. [17]. Briefly, sheep red blood cells (SRBC) (supplied by TCS Biosciences) were washed with a 0.85% saline solution, re-suspended in a gelatin veronol buffer (GVB) and diluted to a standard working concentration of 8×10^6 SRBC per mL. Carp serum was then serially diluted from 0.5% to 7%. A positive control reflecting 100% haemolysis and a sample test were prepared for each serum dilution and incubated for 1 h at 20 °C. Samples were then centrifuged (400 g, 5 min, 4 °C) and kept on ice at 4 °C to stop the lytic reaction. The optical density of 200 μL of supernatant was recorded at 410 nm (Labsystems Multiskan MS plate reader) to measure the haemoglobin release. For each serum dilution, the rate of haemolysis is calculated and the ACH50 (in ACH50 units/mL) was determined.

2.7. Gene expression by real-time PCR

The expression profile of two carp CRP-like genes (*crp1* and *crp2*; [28]) and several carp complement genes (*c1r/s*, *bf/c2*, *c3* and

masp2; [33]) were analysed in the liver (the main production site for CRP), head kidney (a major immune organ) and mid-gut (oral route of β-glucan administration). Complement component genes have been selected based on their functional role and position in the complement pathways i.e. *c1r/s* for classical, *bf/c2* for classical and alternative, *masp2* for lectin, and *c3* because of its central role in the complement cascade [34].

2.7.1. RNA isolation and cDNA synthesis

RNA extraction and purification from liver, head kidney and mid-gut tissues was achieved using the RNeasy Mini Kit (Qiagen, UK) following manufacturer's instructions and the concentration was determined by Nanodrop 1000 (Thermo Scientific, UK). RNA was re-suspended in diethyl pyrocarbonate (DEPC)-treated water (Invitrogen) and then subjected to cDNA synthesis according to manufacturer's instructions (Invitrogen). Briefly, 0.5 μg RNA was added to 0.5 mM dNTPs, 5 mM $MgCl_2$, 5× PCR Buffer II, 25 μM random hexamers, DEPC-treated water and 25 units of Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase. All reactions were carried out using a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems). cDNA samples were then stored at −20 °C.

2.7.2. Real time RT-PCR

A volume of cDNA equivalent to 5 ng of RNA was added to 900 nM of each primer (Table 1) and 1× Power SYBR Green PCR Master Mix (Applied Biosystem) in a final volume adjusted with DEPC-treated water to 20 μL. Thermal cycling conditions (ABI PRISM® 7000 Sequence Detector System, Applied Biosystems) comprised of 2 min at 50 °C, 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. A melting curve was created for each run and checked to ensure a single product was amplified. Results were analysed according to the $2^{-\Delta\Delta Ct}$ method [35] and normalised against the expression of the 40S ribosomal protein S11 reference gene [36]. As the gene expression profiles in the control group were stable throughout the duration of the treatment for all the selected genes and in all the analysed tissues (see Supplementary Figure S1), results are presented as x-fold change in relation to the control treatment.

2.8. Statistical analysis

All data are given as means ± standard error of the mean (SEM). Statistical analysis was carried out using GraphPad Prism v5 and PASW Statistics 18 software. Data were tested for normality and homoscedasticity prior to further analysis. A two-way analysis of variance (ANOVA) and post-hoc Bonferroni's multiple comparisons test have been performed on serum cCRP levels and complement activity to determine significant differences between the treatments at their respective time point during feeding, or between the treatments and their respective time point control group (control fed and PBS injected fish) for the PAMPs injection. Gene expression data were normalised using a log-transformation prior to a one-way ANOVA analysis for the feeding and a two-way ANOVA analysis for the PAMPs injection, followed by subsequent Bonferroni post-hoc test analyses. Significance was defined as $p \leq 0.05$.

3. Results

3.1. Serum cCRP levels

The serum cCRP levels (Fig. 1A) were significantly different between the β-glucan and control diet fed groups only ($F = 10.99$, df = 1, $p = 0.0023$) and also during the time of the treatment ($F = 7.698$, df = 3, $p = 0.0005$). However the time/treatment interaction had no significant ($F = 1.821$, df = 3, $p = 0.1631$) effect on the levels of this acute phase protein. A Bonferroni post-hoc analysis revealed that the basal serum cCRP level was significantly higher ($p < 0.05$) in the β-glucan fed group (9.7 μg/mL) compared to the control fed group (5.2 μg/mL) after 7 days of feeding and cCRP levels in the β-glucan fed fish were always higher than in the control diet group during the whole feeding period.

In contrast, after the PAMPs injection (Fig. 1B), no effect of the treatment, time course or the interaction of these factors on the serum cCRP levels were observed. A Bonferroni post-hoc analysis also revealed that there was no significant differences between all the groups and their respective time point control group (control fed + PBS injected fish).

3.2. Serum ACP activity

In contrast to the effect noted of β-glucan feeding on serum cCRP levels there was no significant difference ($F = 2.413$, df = 1, $p = 0.1316$) of serum ACP activity (Fig. 2A) between the two feeding treatments, or during the time period of the experiment ($F = 1.087$, df = 3, $p = 0.3708$). However a significant ($F = 5.024$, df = 3, $p = 0.0065$) time/treatment interaction, resulted in a significant difference ($t = 3.357$, $p < 0.01$) in ACP activity after 25 days of feeding between the β-glucan fed group (749 ACH50 units/mL) and the control fed group (149 ACH50 units/mL). It is also interesting to note that, as in CRP levels, the serum ACP activity was higher in the β-glucan fed group than in the control fed group after 7 days and became significantly different after 25 days of feeding.

Table 1
Primers used in this study.

Gene	Gene function	Primer type	Sequence 5′–3′	GenBank accession no.	Reference
40S	housekeeping gene	Forward	CCGTGGGTGACATCGTTACA	AB012087	[36]
		Reverse	TCAGGACATTGAACCTCACTGTCT		
crp1	CRP response	Forward	AGCAATGCAACATTTTTCCGTC	JQ010977	[38]
		Reverse	ACTTGCGTCAAAGCCACCCAC		
crp2	CRP response	Forward	GATGCTGCAGCATTTTTCAGTC	JQ010978	[38]
		Reverse	CTCCGCATCAAAGTTGCTCAAAT		
c1rs	classical complement pathway	Forward	CAAGCCCATCTTGGCTCCTGG	AB042609	[33]
		Reverse	GTCCAGATCAAGCGGGGACGT		
bf/c2	classical and alternative complement pathways	Forward	CGGTCATGGGAAAAAGCATTGAGA	AB047361	[33]
		Reverse	GATATCTTTAGCATTTGTCGCAG		
c3	central component of the complement system; marker of the three pathways	Forward	GGTTATCAAGGGGAGTTGAGCTAT	AB016215	[33]
		Reverse	TGCTGCTTTGGGTGGATGGGT		
masp2	MB-Lectine complement pathway	Forward	CAAGCTGTCCAAGGTGATTG	AB234294	[33]
		Reverse	AGCAGTGAGGACCCAGTTGT		

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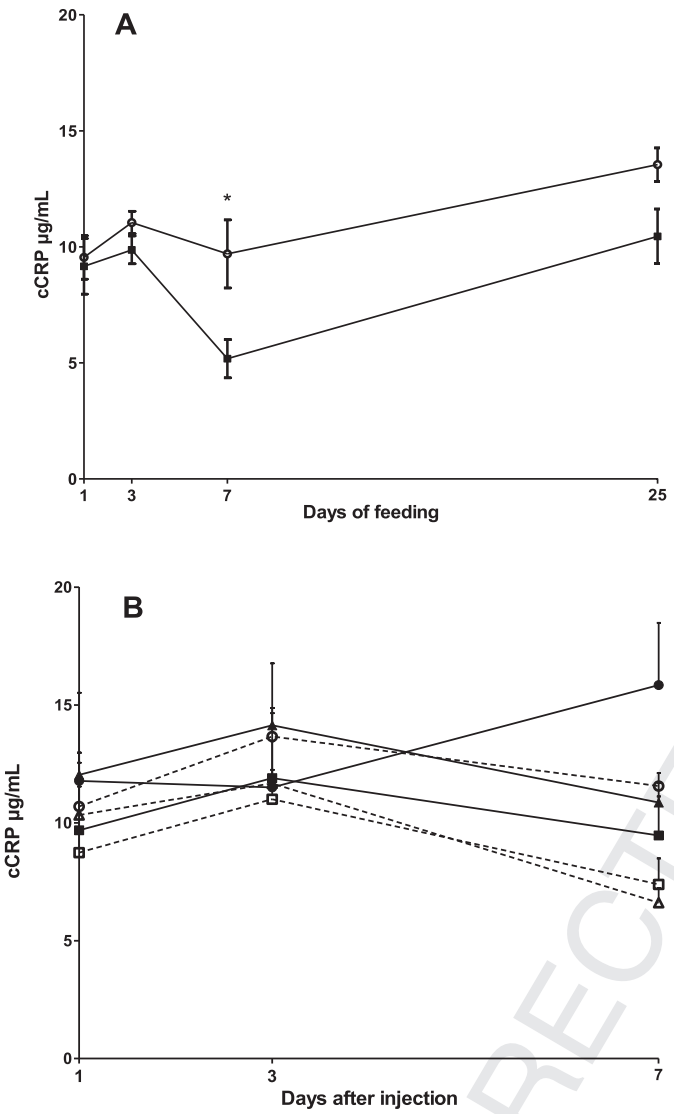

Fig. 1. Carp serum cCRP levels profiles during 25 days of β-glucan feeding (A) followed by PAMPs injections (B). A: ■: control fed group, ●: β-glucan fed group. Mean are ±SEM, $n = 5$. *: Bonferroni's post hoc two-way ANOVA $p \leq 0.05$. B: In black filled symbols, control fed fish injected with: ■: PBS; ●: LPS and ▲: poly(I:C). In blank leaved symbols and in dotted lines, β-glucan fed fish injected with: ⊡: PBS; ⊙: LPS and ▲: poly(I:C). Mean are ± SEM, $n = 4$.

In contrast, serum ACP activity was significantly affected by the PAMPs injection ($F = 4.126$, df = 5, $p = 0.003$) (Fig. 2B) but was not affected either by the time period ($F = 0.4582$, df = 2, $p = 0.6349$) or by the time/treatment interaction ($F = 1.326$, df = 10, $p = 0.2405$). A Bonferroni post-hoc analysis revealed a significant difference ($p \leq 0.01$) in ACP activity at 3 days p.i. between the control fed/LPS injected group (1260 ACH50 units/mL), the control fed/poly(I:C) injected group (1364 ACH50 units/mL) and the control fed/PBS injected group (258 ACH50 units/mL). Also as noted in the serum CRP levels, complement activity was not affected by the PAMPs injection in the β-glucan fed groups over the time period studied.

3.3. CRP and complement related gene expression profiles during 25 days of feeding

The one-way ANOVA analysis revealed that there was a differential effect on the expression profiles of CRP and complement related genes depending upon the diet fed, organ analysed and time period of feeding. In the liver, the gene expression was down-regulated (Fig. 3) after 7 days of feeding ($F = 8.889$, df = 1, $p = 0.0045$) but not after 25 days of feeding ($F = 1.032$, df = 1, $p = 0.3148$). However, in the head kidney, the β-glucan feeding did not significantly affect the expression of the analysed genes either after 7 or 25 days of feeding ($F = 3.293$, df = 1, $p = 0.0758$ at 7 days; $F = 0.3365$, df = 1, $p = 0.5646$ at 25 days). Interestingly, the β-glucan feeding did have a significant effect, mostly up-regulating the CRP and complement gene expression profiles in the mid-gut tissue after 7 days ($F = 4.328$, df = 1, $p = 0.0428$) and 25 days of feeding ($F = 12.75$, df = 1, $p = 0.0008$). However, the effects on the gene expression after 25 days were also dependent on the gene analysed ($F = 10.63$, df = 1, $p < 0.0001$).

Bonferroni's post-hoc analyses have shown that when considering the effect on genes encoding CRP there was a similar differential effect. For example, *crp1* was significantly down-regulated in the liver (0.79 fold, $p \leq 0.05$) and head kidney (0.91 fold, $p \leq 0.01$)

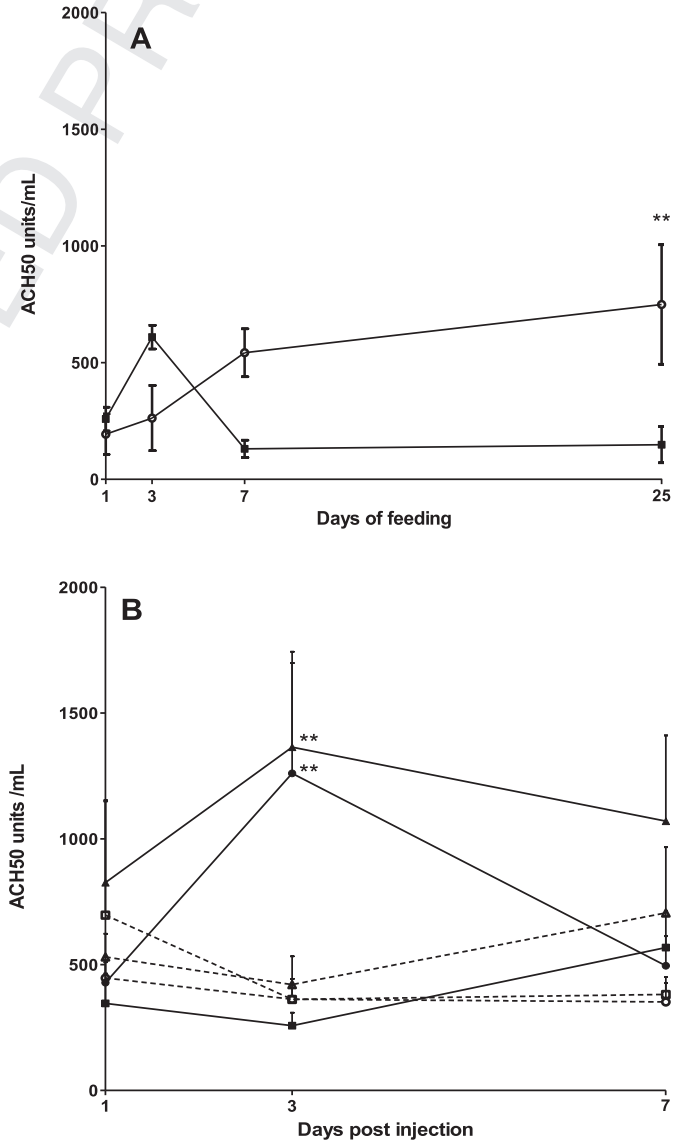

Fig. 2. Carp serum ACP activity profiles during 25 days of β-glucan feeding (A) followed by PAMPs injections (B). A: ■: control fed group, ●: β-glucan fed group. Mean are ±SEM, $n = 5$. **: Bonferroni's post hoc two-way ANOVA $p \leq 0.01$. B: In black filled symbols, control fed fish injected with: ■: PBS; ●: LPS and ▲: poly(I:C). In blank leaved symbols and in dotted lines, β-glucan fed fish injected with: ⊡: PBS; ⊙: LPS and ▲: poly(I:C). Mean are ±SEM, $n = 4$. **: Bonferroni's post hoc two-way ANOVA $p \leq 0.01$.

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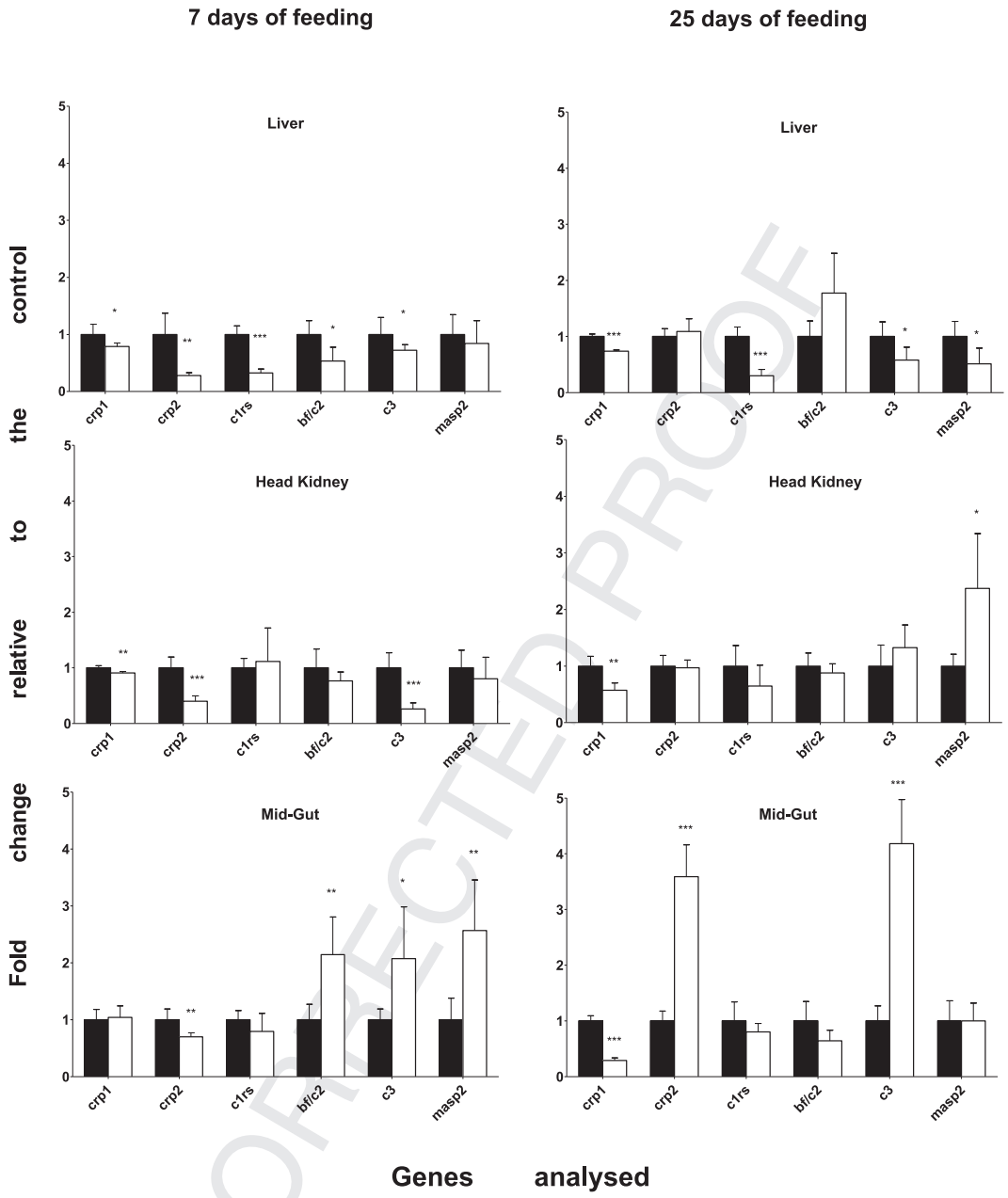

Fig. 3. CRP (*crp1* and *crp2*) and complement (*c1rs*, *bf/c2*, *c3* and *masp2*) related genes expression profiles in the head kidney, mid-gut and liver tissues of carp after 7 or 25 days of control or β-glucan feeding. Expression levels are shown as x-fold change for β-glucan fed fish (white bars) compared to the control fed fish (black bars) for each gene. Mean are ±SEM, $n = 5$. *, ** and ***: Bonferroni's post hoc two-way ANOVA $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ respectively when comparing the data to the control group.

after 7 days of feeding but not in the mid-gut. However, after 25 days of feeding, a down-regulation was observed in all the tissues examined (from 0.29 to 0.74 fold, $p \leq 0.01$ and ≤ 0.001). Interestingly, *crp2* was also significantly down-regulated in the three organs examined but only after 7 days of feeding (0.28–0.7 fold, $p \leq 0.01$ and ≤ 0.001). In addition, although after 25 days of feeding the expression of *crp2* was not affected in the liver and head kidney tissues, there was a significant up-regulation in the mid-gut tissue (3.59 fold, $p \leq 0.001$).

This differential effect was also noted in the complement related genes analysed. For example, although the expression of *c1rs* was not affected in the head kidney or mid-gut tissues, either after 7 or 25 days of feeding with β-glucan, there was a significant down-regulation of this gene in the liver at 7 days (0.32 fold, $p \leq 0.001$) and 25 days (0.3 fold, $p \leq 0.001$). After 7 days of β-glucan feeding, *bf/c2* was also significantly down-regulated in the liver (0.53 fold, $p \leq 0.05$) but was significantly up-regulated in the mid-gut (2.1 fold, $p \leq 0.01$). However, its expression was not significantly affected in any of the three organs analysed after 25 days of feeding. The expression of the *c3* gene which is a common component in all three complement activation pathways was significantly down-regulated in the liver at 7 and 25 days (0.58 fold, $p \leq 0.05$) as well as in the head kidney after 7 days of β-glucan feeding (0.26 fold, $p \leq 0.001$). In contrast, this gene was significantly up-regulated in the mid-gut after 7 days (2.1 fold, $p \leq 0.05$) and 25 days (4.2 fold, $p \leq 0.001$). Finally, the expression of the *masp2* gene was significantly affected in the mid-gut after 7 days of feeding (2.56 fold up-regulation, $p \leq 0.01$), and after 25 days of feeding with

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β-glucan in the kidney and liver (i.e. 2.37 fold up-regulation in the kidney, $p \leq 0.05$; 0.51 fold down-regulation in the liver, $p \leq 0.05$).

3.4. CRP and complement related gene expression profiles after PAMPs injection

The application of the PAMPs (LPS or poly(I:C)) had a differential effect on the genes analysed dependent on the feeding regime and the organ examined. In the liver (Fig. 4), with the exception of *crp2*, the expression of all the analysed genes was significantly affected by the treatment ($F = 4.063–11.37$, df = 5, $p \leq 0.0033$) whilst the time of the treatment did not have a significant affect ($F = 4.636–12.88$, df = 2, $p \leq 0.0139$). In addition, with the exception of *crp1*, the interaction between treatment/time also significantly affected the expression of all of the genes analysed ($F = 2.235–8.646$, df = 10, $p \leq 0.0291$).

Post-hoc analysis revealed a pattern of up- or down-regulations occurring in the control fed fish at 1 day p.i., i.e. genes encoding for C1rs, MASP2 and CRP1 were down-regulated (from 0.15 to 0.52 fold, $p \leq 0.05$) in both LPS and poly(I:C) injected groups, whilst the gene encoding for CRP2 was up-regulated in the poly(I:C) injected group (2.37 fold, $p \leq 0.01$) as was the *c3* gene in the LPS injected group (2.66 fold, $p \leq 0.05$). After 3 days, the expression of genes encoding for C1rs, C3 and MASP2 were up-regulated (up to 3.5–4.5 fold for *c3*, $p \leq 0.01$), although this effect was not maintained after 7 days the expression with the exception of *crp2* where a significant *crp2* up-regulation occurred in LPS and poly(I:C) injected groups (4.5–9.35 fold, $p \leq 0.05$).

At 3 or 7 days p.i. in the β-glucan fed fish the CRP and complement gene expression was similarly affected in the LPS, poly(I:C) or PBS injected groups. However distinct patterns of gene up- or down-regulations were detected at 1 day p.i. in each of the groups.

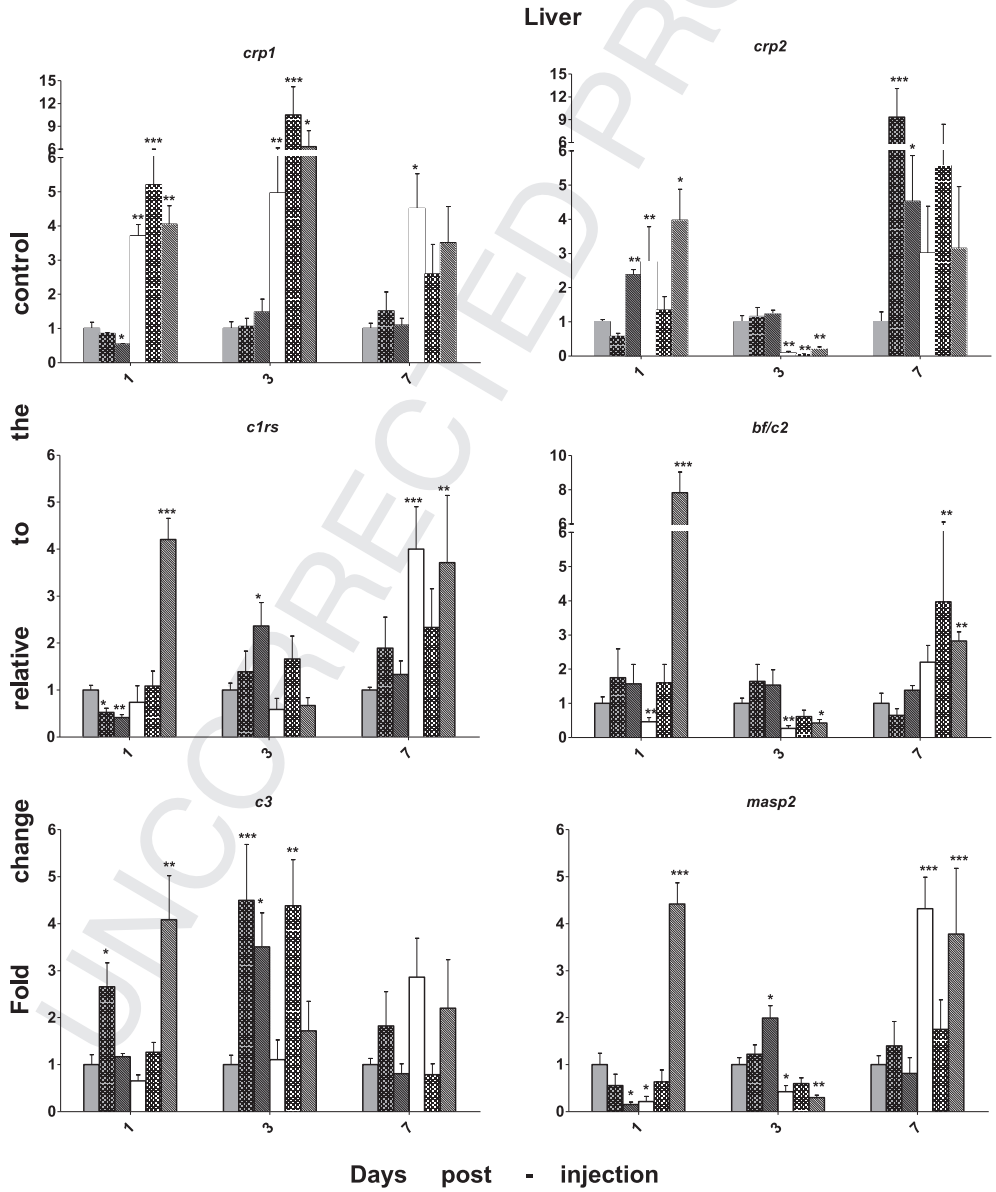

Fig. 4. CRP (*crp1* and *crp2*) and complement (*c1rs*, *bf/c2*, *c3* and *masp2*) related genes expression profiles in the liver tissue of carp at 1, 3 or 7 days post-PAMPs injection. Expression levels of the different treatment group samples are shown as x-fold change compared to the control fed + PBS injected fish samples (grey bars) at each time point. Mean are ± SEM, $n = 4$. Significant differences when comparing the different treatment groups to their respective time point control groups (grey bars) are represented by *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$. ▭: control feed + PBS injection (control group), ▩: control feed + LPS injection ▬: control feed + poly(I:C) injection, ▭: β-glucan feed + PBS injection ▩: β-glucan feed + LPS injection, ▬: β-glucan feed + poly(I:C) injection.

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Whilst in the PBS injected group several up- or down-regulations in gene expression were observed (i.e. down-regulation of *bf/c2* and *masp2*, $p \leq 0.05$; and up-regulation of *crp1* and *crp2*, $p \leq 0.01$), only the gene expression of *crp1* was affected (5.2 fold up-regulation, $p \leq 0.001$) in the LPS injected group and, most interestingly, all the genes were up-regulated in the poly(I:C) injected fish (from 3.97 to 7.8 fold, $p \leq 0.05$). At 3 days p.i. however, *bf/c2*, *masp2*, *crp2* gene expressions (0.03–0.61 fold, $p \leq 0.05$) were down-regulated, while the expression of the *crp1* remained up-regulated (4.9–10.5 fold, $p \leq 0.05$). Finally, at 7 days p.i. the expression of genes encoding for CRP2, Bf/C2, C3 and MASP2 were significantly up-regulated in the PBS, LPS and poly(I:C) injected groups.

In the head kidney (Fig. 5), in contrast to the liver, only the expression of *c1rs*, *bf/c2* and *masp2* was significantly affected by the injections ($F = 11.03–21.7$, df = 5, $p < 0.0001$), whilst the time effect was significant for all the genes analysed ($F = 12.08–63.98$, df = 2, $p < 0.0001$). The interaction treatment/time also had a significantly effect on the gene expression of *crp1*, *c1rs*, *bf/c2* and *masp2* ($F = 3.508–10.96$, df = 10, $p \leq 0.0013$) but not the expression of genes encoding for CRP2 or Bf/C2 ($F = 0.918$ and 1.777, df = 10, $p \geq 0.0873$).

In fish fed diets lacking β-glucan, the expression of the analysed genes was not affected until 3 days p.i., with the exception *crp1* and *crp2* which were both up-regulated (1.77 and 2.37 fold respectively, $p \leq 0.05$ and $p \leq 0.01$) in the group injected with poly(I:C) at 1 day p.i. The post-hoc analysis also revealed a pattern of significant gene up-regulation occurring at 7 days p.i. For example, *c1rs*, *bf/c2*, *c3*, *masp2* and *crp2* were all significantly up-regulated and most

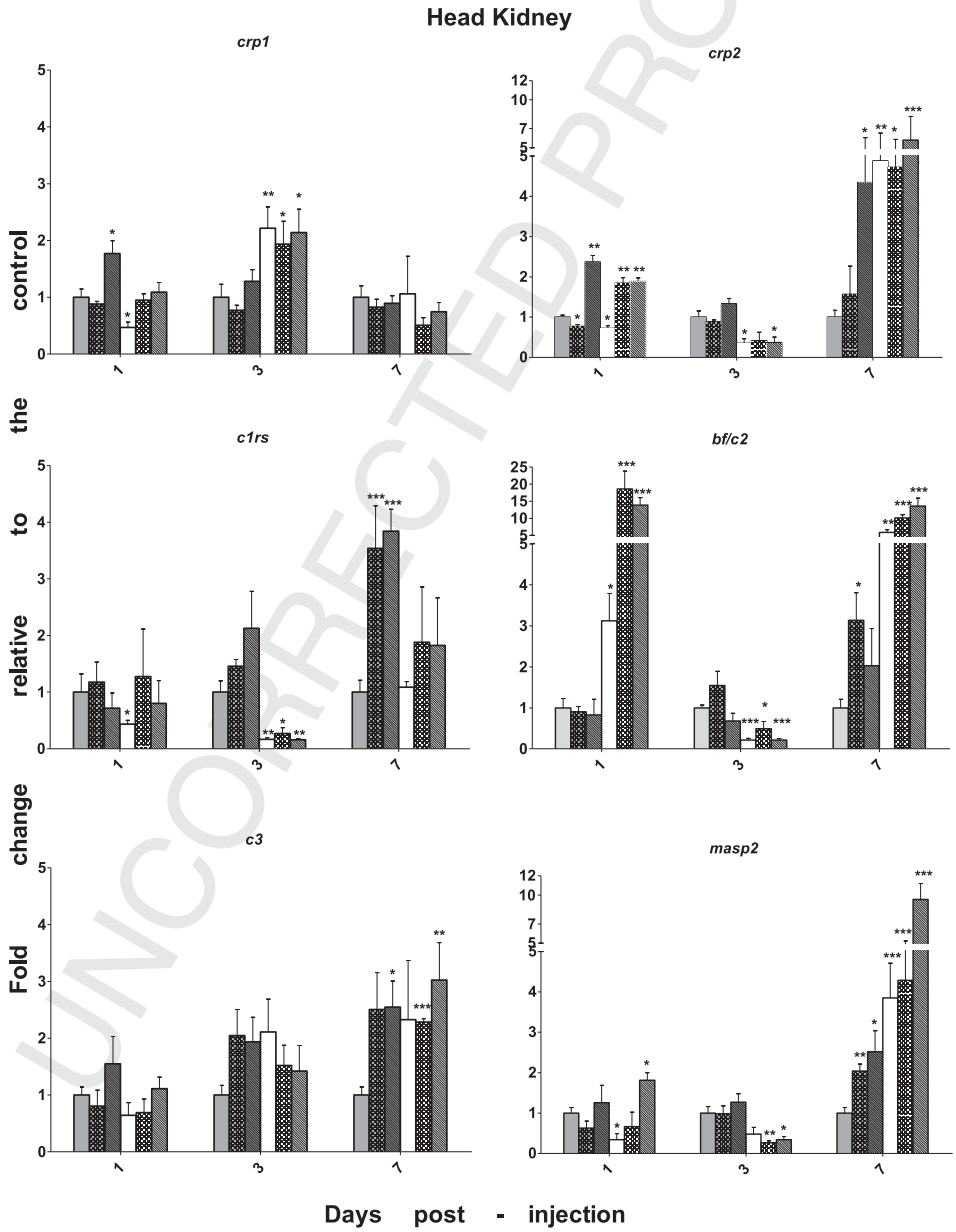

Fig. 5. CRP (*crp1* and *crp2*) and complement (*c1rs*, *bf/c2*, *c3* and *masp2*) related genes expression profiles in the head kidney tissue of carp at 1, 3 or 7 days post-PAMPs injection. Expression levels of the different treatment group samples are shown as x-fold change compared to the control fed + PBS injected fish samples (grey bars) at each time point. Mean are ± SEM, $n = 4$. Significant differences when comparing the different treatment groups to their respective time point control groups (grey bars) are represented by *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$. ▭: control feed + PBS injection (control group), ▩: control feed + LPS injection ▬: control feed + poly(I:C) injection, ▭: β-glucan feed + PBS injection ▩: β-glucan feed + LPS injection, ▬: β-glucan feed + poly(I:C) injection.

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predominantly receiving an injection with LPS or poly(I:C) (for instance, up to 4.33–4.88 fold for *crp2*, $p \leq 0.05$). In the β-glucan fed groups, time also appeared to affect the expression of the analysed genes. Whilst at 1 day p.i. the gene expression was not affected except a significant up-regulation of *bf/c2* (3.1–18.6 fold, $p \leq 0.05$ and $p \leq 0.001$), after 3 days, *c1rs*, *bf/c2*, *masp2* and *crp2* were significantly down-regulated (0.16–0.49 fold, $p \leq 0.05$), whilst *crp1* was up-regulated (1.93–2.21 fold, $p \leq 0.05$). In contrast, at 7 days p.i. a pattern of significant up-regulations was detected in four of the analysed genes i.e. *c3*, *bf/c2*, *masp2* and *crp2*, either in PBS, LPS or poly(I:C) injected groups (5.8–13.6 fold up-regulations for *bf/c2*, $p \leq 0.01$ and 4.72–5.79 fold up-regulations for *crp2*, $p \leq 0.05$).

Finally, in the mid-gut tissue (Fig. 6), injections significantly affected the expression of *crp1*, *crp2* and *bf/c2* genes ($F = 3.939$–6.682, df = 5, $p \leq 0.0073$) but not the expression of the genes encoding for C1rs or MASP2. As in the liver and head kidney, the time of treatment also had a significant effect on all the genes analysed ($F = 3.194$–15.46, df = 2, $p \leq 0.0488$), except for *crp1*. In addition, with the exception of *c1rs*, the interaction between treatment/time also significantly affected the expression of all the genes ($F = 2.508$–5.499, df = 10, $p \leq 0.0148$).

In the fish fed the control diet, only up-regulations were detected and the post-hoc analysis revealed distinct patterns of gene expression for the LPS and poly(I:C) injected groups. In the LPS injected fish, no significant changes in gene expression were detected after 1 day but *c3* was significantly up-regulated after 3 days (2.8 fold, $p \leq 0.05$) and *c1rs* and *masp2* after 7 days (2.3 and 1.9 fold respectively, $p \leq 0.05$). In contrast, in the poly(I:C) injected group, *c3* and *crp1* were significantly up-regulated at 1 day p.i. (4.7 and 2.8 fold respectively, $p \leq 0.001$) as well as *c1rs* after 7 days (2.6 fold, $p \leq 0.05$). In the β-glucan fed groups, several significant up- or down-regulations were detected at 1 day p.i., for example up-regulation of *bf/c2* in the LPS and poly(I:C) injected groups (6.5 and 4 fold respectively, $p \leq 0.05$), down-regulation of *masp2* in the

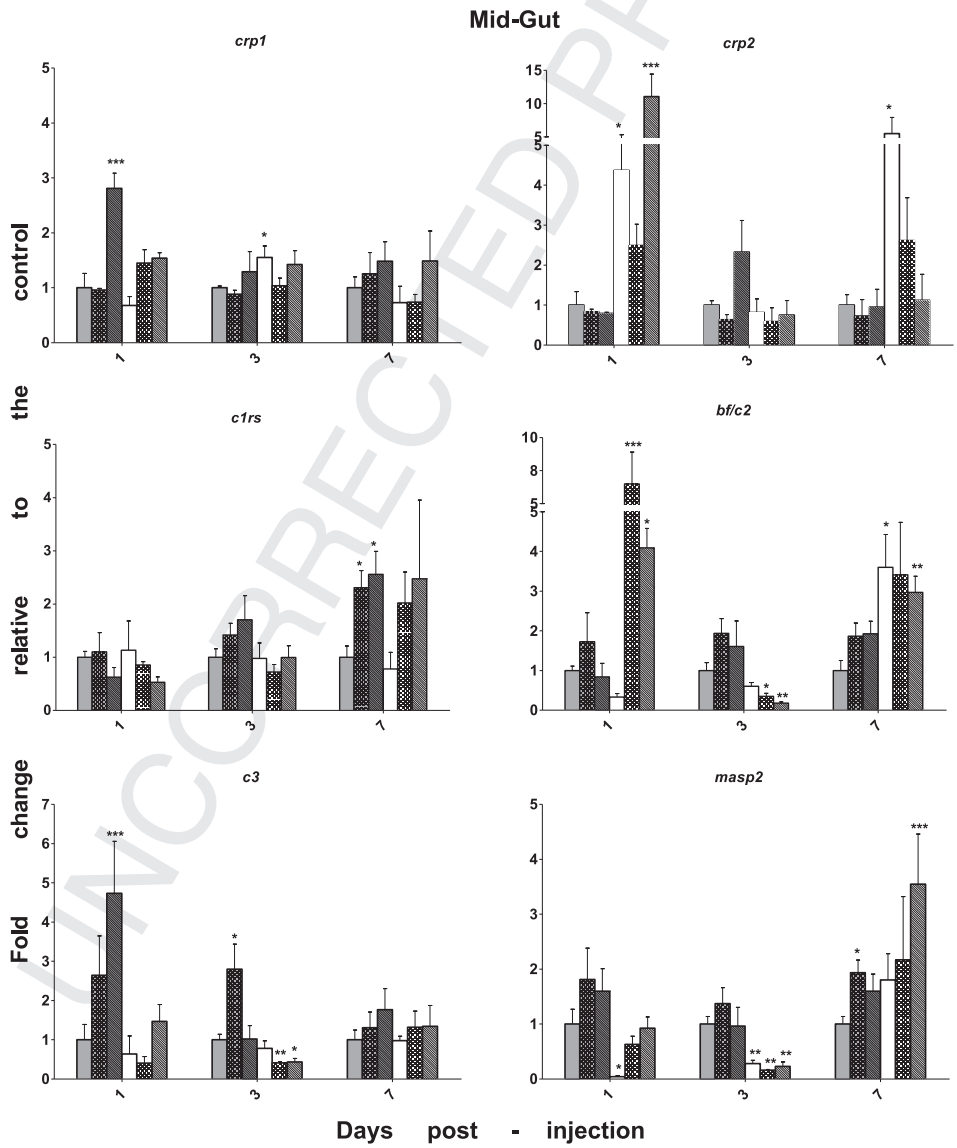

Fig. 6. CRP (*crp1* and *crp2*) and complement (*c1rs*, *bf/c2*, *c3* and *masp2*) related genes expression profiles in the mid-gut tissue of carp at 1, 3 or 7 days post-PAMPs injection. Expression levels of the different treatment group samples are shown as x-fold change compared to the control fed + PBS injected fish samples (grey bars) at each time point. Mean are ± SEM, $n = 4$. Significant differences when comparing the different treatment groups to their respective time point control groups (grey bars) are represented by *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$. : control feed + PBS injection (control group), : control feed + LPS injection : control feed + poly(I:C) injection, : β-glucan feed + PBS injection : β-glucan feed + LPS injection, : β-glucan feed + poly(I:C) injection.

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PBS injected group (0.04 fold, $p \leq 0.05$) and up-regulation of *crp2* in the PBS and poly(I:C) injected groups (4.4 and 11 fold respectively, $p \leq 0.05$). After 3 days, down-regulation of the genes encoding for *bf/c2*, *c3* and *masp2* were observed in the three β-glucan fed groups (0.16–0.78 fold) whilst at 7 days several significant up-regulations were detected in the PBS and poly(I:C) injected groups i.e. *bf/c2* in the PBS and poly(I:C) injected groups (3.6 and 2.9 fold respectively, $p \leq 0.05$), *masp2* in the poly(I:C) injected group (3.5 fold, $p \leq 0.001$), and *crp2* in the PBS injected group (5.5 fold, $p \leq 0.05$).

4. Discussion

4.1. Dietary β-glucan effects on carp immune acute phase proteins

Whilst oral administration of 0.1% MacroGard® enriched diet significantly increased serum CRP levels and serum alternative complement activity, this was not completely reflected within the CRP and complement related gene expression profiles obtained.

Although CRP is mainly synthesised in the liver in mammals [37], the two CRP-like genes analysed in this study revealed different expression profiles in carp liver and the head kidney, and perhaps more importantly in the mid-gut tissue where significant down-regulation of *crp1* gene and up-regulation of *crp2* gene were observed. Interestingly, previous studies [17] reported significant down-regulation of *crp2* in the mid-gut and head kidney tissues of carp after 14 days of oral stimulation with 0.1% MacroGard®. These contradictory results support previous observations suggesting a possible difference in the biological activity of the related isoforms of this acute phase protein [38] which could also be time and stimulus dependent. Also serum CRP levels did not appear to correlate to the observed down-regulation of the expression of CRP related genes. This might be explained if the levels of free-phase CRP had reached an optimal circulating concentration after 7 days of feeding and therefore the CRP related genes were down-regulated to bring a rapid return to homeostasis as described by Lund and Olafsen [39]. Alternatively there could be more than two CRP related genes in common carp genome encoding for this important innate immune protein, and indeed seven CRP-like genes have been proposed in zebrafish [28]. However it should be noted that although the literature on the effects of β-glucan on the CRP acute phase protein profiles is inconsistent [2], the down-regulation observed for CRP related genes is in agreement with other studies conducted with immunostimulants. For instance, Liu et al. [40] showed that the synthesis of CRP in rainbow trout hepatocytes, head kidney macrophages and spleen lymphocytes significantly decreased after intramuscular injection of turpentine oil used as an acute phase immune response inducer.

There was also an apparent discrepancy between the high serum ACP activity in the β-glucan fed fish and the absence of a significant effect on the related gene expression *bf/c2* and *c3*, except in the mid-gut tissue. This might be explained by the polymorphic complexity of the complement system in common carp [34,41] and the choice of the primers used [33] which may not have detected the appropriate gene isoforms responsible for the Bf/C2 and C3 proteins expression. The results however do suggest that based on the gene expression, the lectin and classical complement pathways (*c1rs* and *masp2* respectively) may not have been actively stimulated by oral administration of β-glucan, except in the mid-gut tissue where *masp2* was up-regulated. This is supported by previous studies which revealed that oral administration of β-glucan did not affect serum complement activity in rainbow trout fed two weeks with β-glucan [42], in large yellow croaker *Pseudosciaena crocea* fed with 0–0.18% β-1,3-glucan enriched diet for 8 weeks [43] and in common carp either intraperitoneally injected, bath immersed or orally administered with β-glucan [9,10]. Nevertheless, our results are in accordance with those from our previous study showing increased complement activity in the serum of carp orally stimulated with 0.1% MacroGard® [17] and with the findings of several studies showing increased complement activity in sea bass *Dicentrarchus labrax* fed with a 2% β-1,3/β-1,6-glucan diet over a 2-week period [44], in rohu *Labeo rohita* injected with different doses of β-glucan (0, 5, 10 and 15 mg/kg fish body weight) [6] or in common carp intraperitoneally injected with 2–10 mg/kg of different polysaccharides (curdlan, inulin, krestin, laminaran, lentinan, levan, schizophyllan, scleroglucan, yeast glucan and zymosan) [16].

Interestingly, in contrast to the down-regulation of genes encoding CRP and complement occurring in the head kidney and liver, numerous gene up-regulations were noted in the mid-gut, either after 7 or 25 days of feeding, as observed in previous studies focused on other immune related genes [28] and apoptosis related genes [45] expression profiles. These results support the hypothesis that, as the β-glucan was incorporated into food, its stimulating effects were initially localised in the mid-gut. For instance, it has been shown in *S. salar* that β-glucan was absorbed by the posterior part of the gut [23]. However, in this study, early effects, mostly gene down-regulations, were detected in the liver and head kidney, suggesting that the β-glucan may also have some systemic effects that are organ and time dependent. The differential response of organs to the oral administration of this immunostimulant has also been noted in our previous studies on inflammatory genes such as TNF-α, IL-1β, IL-6 and IL-10 in the mid-gut and the head kidney [28] and iNOS as well as apoptosis related genes [45].

4.2. Combined action of β-glucan and PAMPs on carp immune acute phase proteins

Interestingly, intraperitoneal injections of LPS or poly(I:C) did not have significant effects on serum CRP levels and ACP activity in β-glucan fed fish but only in fish fed the control diet. However, CRP and complement related gene expression profiles were significantly affected in the liver, head kidney and mid-gut in both feeding groups.

Whilst LPS injection increased serum CRP and complement responses in the control fed fish this was not observed in fish fed with β-glucan. The observed increase in CRP levels in the fish fed the control diet was associated with an up-regulation of *crp2* gene occurring in the liver at 7 days p.i. Jorgensen et al. [25] also reported a similar up-regulation in serum amyloid A (SAA) gene expression in the hepatocytes of *S. salar* stimulated with LPS. Although CRP is known to bind to LPS [26,46,47], our results may suggest that a CRP acute response was not triggered in the control fed fish by LPS injection. This is in accordance with previous studies conducted in common carp injected with LPS (3 mg/kg) where an absence of a typical large CRP acute phase reaction was noted by MacCarthy et al. [26].

Although LPS injection induced a significant increase in serum ACP activity at 3 days p.i. in the control fed fish, this was not associated with the expression profile of *bf/c2*, an alternative complement pathway related gene, which suggests that the primers utilised did not detect the appropriate gene isoform responsible for the Bf/C2 protein expression. However, *c3* up-regulations were observed at 3 days p.i. in the liver and in the mid-gut, which does indicate that the complement cascade was activated. In addition, the up-regulation of complement related genes in the head kidney (*c1rs*, *c2* and *masp2*) and in the gut (*c1rs* and *masp2*) may suggest that both the classical and lectin complement pathways were triggered in response to LPS injection in the fish fed the control diet but this activation occurred later than 7

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days post-injection. These results are in contradiction with previous studies performed on serum acute phase protein profiles in common carp intraperitoneally injected with LPS from *A. hydrophila* where the complement was not activated [48], but is in accordance with other studies performed on *Pagrus auratus* [49], *Danio rerio* [50] and in *Oncorhynchus mykiss* [51] challenged with LPS from *A. salmonicida*. In addition, the considerable ranges of up- or down-regulations detected in the β-glucan fed fish for the complement related genes could be explained as a feed-back control exerted on the complement component production in order to maintain the stimulation of the complement response and especially the serum ACP activity. This could indicate why no difference in ACP activity was detected in this study as well as in previous studies conducted on common carp stimulated with β-glucan and LPS via either bath immersion, oral administration or intraperitoneal injection [10,48].

Poly(I:C) injection did not increase serum CRP levels in the control fed fish even though CRP related genes were often up-regulated in the three organs examined at 1 and 7 days p.i. These results suggest that, whilst the poly(I:C) stimulated the production of CRP in the head kidney and liver, the resulting protein isoforms were either not encoded by the CRP genes monitored and/or the encoded protein was not recognised by the anti-cCRP antibody used in the ELISA assay performed on the serum samples [29]. Furthermore, our previous unpublished studies have shown that CRP levels were increased up to 10 fold in common carp following CyHV3 infection. In contrast, the injection of poly(I:C) significantly increased the ACP activity up to 1364 ACH50 units/mL, but the ACP related gene expression (*bf/c2* and *c3*) was not affected. Interestingly, the effects of the poly(I:C) injection were first observed in the liver and then in the other tissues examined at 7 days p.i. This suggests that poly(I:C) injection had an effect on complement response in carp and this effect was organ and time dependent. Although information about poly(I:C) effects on acute phase protein profiles in fish is limited, these results are in accordance with previous studies performed on *Pseudosciaena crocea* [52,53] and *S. salar* [54] where poly(I:C) induced an acute phase profile in the expression of related genes. Regarding β-glucan fed fish, poly(I:C) did not have an effect on serum acute phase protein levels. However, CRP and complement related gene expression was more affected than in the control fed fish. An interesting pattern of up-regulation at 1 day p.i. then down-regulation at 3 days p.i., and up-regulation at 7 days p.i. was detected in the three different organs for three of the six analysed genes i.e. *bf/c2*, *masp2* and *crp2* in the head kidney and liver, and for *bf/c2* but only in the mid-gut. In comparison, in the fish fed the control diet, an up regulation of those genes analysed occurred at 3 days p.i. The oral administration of β-glucan would therefore appear to induced a sufficient immunostimulation to cope with the effects of the injection of poly(I:C) in the first few days, then the production of acute phase proteins was *de novo* stimulated, which would explain the up-regulation of genes noted at 7 days p.i. In addition to this particular gene regulation pattern found in β-glucan fed fish, it is interesting to note that the poly(I:C) had a greater effect on CRP and complement related gene up- or down-regulations across the three organs examined. This may suggest that MacroGard® stimulated CRP and complement responses to poly(I:C) injection and would support previous studies conducted on rainbow trout which have shown that poly(I:C) combined with zymosan or β-glucan significantly induced up-regulation of inflammatory cytokines such as IL-1β, TNF-α, IL-6 and IL-10 [55]. These results highlight the complexity of the carp immune acute phase response [36] and the fact that the determination serum acute phase protein profile may not be enough to estimate the degree of the immune response [38]. To a greater extent, mRNA level of a given gene may not systematically reflect protein level and activity due to post-transcriptional and transductional regulation mechanisms. In addition, the tetraploidy nature of the common carp and the preservation of multiple isoforms of innate immune proteins in fish [41,56], makes the selection of the genes to analyse harder.

In conclusion, results suggest that a 25 day period of β-glucan oral administration induced and enhanced an immune response in carp and subsequent LPS and poly(I:C) injections significantly affected carp CRP and complement responses. In addition, the administered dose of β-glucan (6 mg β-glucan per kg of fish body weight) appeared to induce CRP and complement responses in common carp, thus avoiding situations where the immunostimulant is administered at too low or high doses resulting in a failed enhancement of the immune defences [2,5,9,10,18,19,43]. Furthermore, our results also add to the growing body of evidence that the immunostimulation induced by β-glucan may play a significant response in disease protection in aquaculture.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2014.05.008.

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